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Screening of proteins interacting with Gqα by yeast two-hybrid technique in the antenna of *Helicoverpa armigera* (Lepidoptera: Noctuidae)

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Abstract: G proteins are important signalling components. Many findings indicated that members of Gq family were functionally diverse than previously thought. In order to determine the exact functions of the α subunit of Gq family of G proteins (Gqα) in signal transduction pathway, Gqα was used as the bait to screen the antenna-cDNA library of the cotton bollworm *Helicoverpa armigera* by the two-hybrid system. More than ten proteins including pheromone binding protein, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ribosomal protein S20, cytochrome oxidase subunit Ⅰ, allatotropin neuropeptide precursor (AT2), translationally controlled tumor protein (TCTP), humoral lectin prepropeptide and glycosylasparaginase were found to interact with Gqα, suggesting that Gqα plays diverse roles in signal transduction. Among the diverse functions, Gqα is most likely to be involved in the pheromone detection among olfactory signalling. The findings may provide a primary basis for future construction of an integrated signalling network in the moth.

Key words: *Helicoverpa armigera*; Gqα; antenna cDNA library; yeast two-hybrid technique; signal transduction; pheromone binding protein

1 INTRODUCTION

Heterotrimeric G-protein consists of an α-subunit which binds and hydrolyses guanosine triphosphate (GTP), a β- and a γ-subunit (Hamm, 1998). The β- and γ-subunits of the heterotrimeric G-proteins form an undissociable complex and represent a functional unit, and α-subunit defines the protein's identity. More than twenty G-protein α-subunits, five β-subunits and twelve γ-subunits had been described in the mammalian system (Offermanns, 2003). Ten G-protein α-subunits, seven β- subunits and one γ-subunit had been identified in plants (Mason and Botella, 2000). Six G-protein β- subunits, three γ-subunits and two γ-subunits were found in *Drosophila melanogaster*, and twenty G-protein α-subunits, two β- subunits and two γ-subunits in *Caenorhabditis elegans* (Brody and Cravchik, 2000). G proteins can be divided into four subfamilies according to the properties of α-subunits which fall into four categories based on structural and functional homologies in mammals: Gi/o, Gq, G12 and Gs (Offermanns, 2003).

G proteins are important signalling components for receptors with seven transmembrane domains (7TMRs) (Marty and Ye, 2010). In humans, G protein-coupled receptors mediate responses to light, odor, taste, hormones and neurotransmitters (Dohlman, 2002). However, more and more evidence confirmed non-canonical functions of heterotrimeric G proteins independent of 7TMR coupling, that is, G proteins are found to play important roles in receptor tyrosine kinase signaling, regulation of oxidant production, development and cell migration through physical and functional interaction with proteins other than 7TMRs (Marty and Ye, 2010).

Gq family members bind not only phospholipase C, but also novel candidate effector proteins, various regulatory proteins, and a growing list of scanfolding adaptor proteins. Many findings demonstrated that Gq family members regulated both overlapping and distinct signalling pathways, suggesting that they are

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more functionally diverse than previously thought (Hubbard and Hepler, 2006). The α subunit of Gq family of G proteins (Gqα) was found broadly expressed in *Homarus americanus*, suggesting that it plays a role in a wide variety of signal transduction events, especially in olfactory transduction (McClintock *et al.*, 1997). In *Bombyx mori*, Gqα and Gsα were thought to mediate a subset of odorant response, respectively (Miura *et al.*, 2005).

So far, some G proteins have been studied in several invertebrates, such as Gqα and Gsα in *H. americanus*, Gqα in *Lymnaea stagnalis Drosophila melanogaster*, Goα in *Locusta migratoria*, Gqα in, Goα in *Manduca sexta*, and Gqα, Goα and Gsα in *B. mori* (Raming *et al.*, 1990; Horgan *et al.*, 1995; Knol *et al.*, 1995; Talluri *et al.*, 1995; McClintock *et al.*, 1997; Xu *et al.*, 1997; Miura *et al.*, 2005). However, little is known about Gqα in *Helicoverpa armigera*, a disastrous pest for many crops around the world. Elucidating the function of G protein and the mechanism of signal transduction might provide a primary basis for future construction of an integrated signalling network and give the theoretical ways to develop the effective attractant and deterrent, and mind new ideas and approaches for potent control of cotton bollworm. Our previous study indicated that Gqα did not directly interact with sensory neuron membrane protein (Su *et al.*, 2010). In this study, yeast two-hybrid system was used to screen the binding proteins to Gqα from *H. armigera* antenna-cDNA library.

2 MATERIALS AND METHODS

2.1 Insects and tissues

H. armigera were reared in the laboratory on an artificial diet at 28℃, 60% relative humidity, exposed to a 16L∶8D photoperiod. Antennae were dissected from adults of the moth within 24 h after emergence.

2.2 RNA isolation and cDNA synthesis

Total RNA was isolated from 10 mg of antennae from *H. armigera* with Trizole (Invitrogen, USA), following the operation instructions. The first-strand cDNA was synthesized with oligo (dT) primers and Reverse Transcription System A3500 (Promega, USA).

2.3 Construction of a DNA-BD fusion

Because pGBKT7 vector has the restriction sites *Eco*R Ⅰ and *Pst* Ⅰ, we generated the gene fragment (Gqα) by PCR using primers (Y-GqS/GqA) which contained the desired restriction sites.

Y-GqS: 5′-TCG $\underline{GAATTC}$ATGGAGTGCTGCAT
*Eco*R Ⅰ
GTCGGAGGA-3′

Y-GqA: 5′-GTC $\underline{CTGCAG}$TTAGACGAGGTTGT
Pst Ⅰ
ACTCCTTAAGGT-3′

Gqα was amplified with the specific primers (Y-GqS/GqA) and the Pyrobest DNA polymerase (TaKaRa, JAPAN) according to the procedure described above. DNA-BD vector (pGBKT7) and the PCR product of Gqα were digested with *Eco*R Ⅰ and *Pst* Ⅰ, respectively. After the recovery of the desired fragments by electrophoresis and the purification of the desired fragments with the DNA Purification Kit (Omega, USA), pGBKT7 vector and Gqα insert were ligated. The ligation mixture was transformed into *Escherichia coli*. We identified the insert-containing plasmid (Gqα-pGBKT7) by restriction analysis and PCR. The *E. coli* of the positive plasmid was sequenced to make sure the open reading frame was right.

2.4 Transcriptional activation test, elimination of the background growth and toxicity test of the DNA-BD fusion

Yeast strain AH109 was transformed with the hybrid construct (Gqα-pGBKT7) using a small-scale transformation protocol according to the BD Matchmaker™ Library Construction & Screening Kits User Manual.

Two resolutions were used to eliminate the background growth of the bait strain on -His medium. One was adding 3-AT to the selection medium, the other was using SD/-Ade/-His/Leu/-Trp medium for the library screening. In this study, the latter one was chosen.

In order to test toxicity of the DNA-BD fusion, the growth rate in liquid culture of AH109 cells transformed with the "empty" DNA-BD vector was compared with that of AH109 cells transformed with the DNA-BD/bait plasmid.

2.5 Generation of an antenna-cDNA library

RNA isolation from the antenna was conducted with Trizole according to the protocol described above. First-strand cDNA was synthesized using an Oligo (dT) primer, and dscDNA was amplified by long distance PCR (LD-PCR) and purified with a BD Chroma Spin™ TE-400 Column according to the BD Matchmaker™ Library Construction&Screening Kits User Manual.

2.6 Construction and screening of a two-hybrid library

pGADT7-Rec which expresses a protein of interest as a GAL4 activation domain (GAL4 AD) fusion containing a hemagglutinin (HA) epitope tag for easy identification with Clontech ' s HA-tag polyclonal antibody, was used to construct a two-hybrid library. The two-hybrid library was screened by cotransformation. This procedure is quick and efficient. We prepared the competent yeast cells with the LiAc method (Sambrook, 1989). After the preparation, the yeast strain AH109 was cotransformed with dscDNA, pGADT7-Rec and Gqα-pGBKT7. The cotransformation mixture was spread on TDO (SD/-His/-Leu/-Trp) and QDO (SD/-Ade/-His/-Leu/-Trp) plates (150 μL cells/150 mm plate). At the same time, the yeast strain AH109 was also cotransformed with SV40 Large T PCR fragment, pGADT7-Rec and pGBKT7-53, which was the positive control. The negative control was the yeast strain AH109 cotransformed with SV40 Large T PCR fragment, pGADT7-Rec and pGBKT7-Lam.

2.7 Analysis of the positive interactions

The positive colonies on SD dropout plates were restreaked 2 – 3 times to segregate the AD/library plasmids, then the well-isolated colonies were transferred to SD/-Ade/-His/-Leu/-Trp plates containing X-α-Gal to verify that they maintained the correct phenotype and to test the expression of *MEL1*. To identify the gene (and thus protein) responsible for a positive two-hybrid interaction, the gene was rescued by plasmid isolation. The plasmids isolated from yeast were transformed into *E. coli* and selected on LB/Amp. The AD plasmid insert was sequenced directly and the sequence was compared with that of other proteins in GenBank.

3 RESULTS

3.1 Construction of a DNA-BD fusion

The insert-containing plasmid (Gqα-pGBKT7) was identified by both restriction analysis and PCR. The positive plasmid was sequenced in the sequencing company. The insert sequence was aligned with that of Gqα submitted to GenBank and found to be right.

3.2 Transcriptional activation test and toxicity test of the DNA-BD fusion

AH109 was transformed with the hybrid construct (Gqα-pGBKT7). The results showed that all the transformant colonies growing on SD/-Trp were white, and did not grow on SD/-Ade/-Trp, but grew on SD/-His/-Trp.

The toxicity test of DNA-BD fusion showed that after 24 h of shaking cultivation in the liquid culture SD/-Trp/Kan (20 μg/mL), OD_{600} of pGBKT7 was 1.339, and OD_{600} of pGBKT7-Gqα was 1.236. Both values were ≥0.8, so the DNA-BD fusion was evaluated as nontoxic.

3.3 Generation of an antenna-cDNA library

The isolated total RNA of *H. armigera* antenna was electrophoresised on the denaturing formaldehyde/agarose gel to estimate its integrity. The results showed that the 28S and 18S ribosomal RNA were bright and integrated. OD_{260} of RNA solution was 0.023, and OD_{280} was 0.012, and the ratio of OD_{260} to OD_{280} was 1.917. The concentration of RNA solution was 0.23 μg/μL, indicating that the isolated RNA has high purity and integrity and can be used to construct the antenna cDNA library. First-strand cDNA was synthesized using an Oligo (dT) primer, and the dscDNA was amplified by long distance PCR (LD-PCR) and analyzed on a 1.2% agarose/EtBr gel. It appeared as a moderately strong smear from ≥0.5 kb to 5 kb, and there was an obvious band near 1.0 kb. The constructed library met the requirement for the future screening of the related proteins.

3.4 Construction and screening of a two-hybrid library

The cotransformation mixture was spread on TDO and QDO plates which were incubated at 30℃ for at least 5 d. The small, pale colonies that appeared after 2 d but never grew to > 1 mm in diameter were ignored. On TDO medium grew many white colonies, and on QDO medium just grew one robust and white colony. The colonies growing on TDO medium were replicated onto QDO medium and incubated at 30℃ for at least 3 – 8 d. The colonies of the positive control and negative control both grew robustly on SD/-Leu/-Trp, some robust colonies of the positive control grew on QDO medium, but no colony of the negative control appeared on QDO medium.

3.5 Analysis of the positive interactions

The well-isolated colonies were transferred to fresh SD/-Ade/-His/-Leu/-Trp/X-α-Gal plates (200 μL of 4 mg/mL X-α-Gal in DMF was used to QDO medium in 150 mm-plate). The colonies of the positive control turned blue on SD/-Ade/-His/-Leu/-Trp/X-α-Gal medium and several target colonies turned blue (Fig. 1). The colonies on QDO medium which turned blue on SD/-Ade/-His/-Leu/-Trp/X-α-Gal were cultured in the liquid YPDA and then the plasmid DNA was isolated from yeast, and then transformed into *E. coli* strain DH5α. The colonies growing on LB/Amp were cultured and the plasmid DNA was isolated and sequenced. The sequences were compared with those in GenBank, which showed that the proteins interacting with Gqα included pheromone binding protein (PBP) of *H.*

armigera and the protein highly similar to pheromone binding protein-2 (PBP-2) of *Heliothis virescens*, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ribosomal protein S20, cytochrome oxidase subunit I gene, allatotropin neuropeptide precursor (AT2), translationally controlled tumor protein (TCTP), humoral lectin prepropeptide and glycosylasparaginase (Table 1).

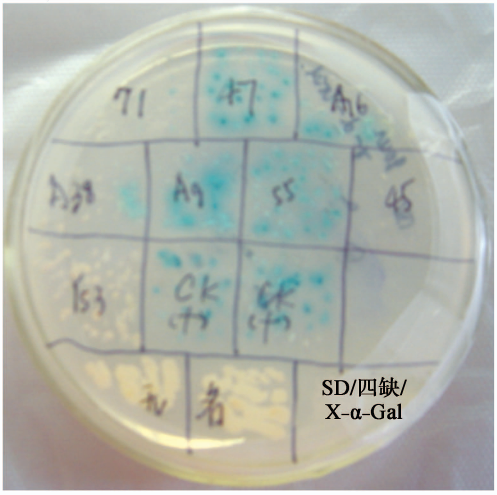

Fig. 1 Proteins binding with Gqα screened by nutritional markers and blue/white colours in *Helicoverpa armigera*
CK(+): Positive control; Blue clones: Positive clones; White clones: Negative clones.

Table 1 Blast results of the proteins binding with Gqα in *Helicoverpa armigera* in GenBank

Proteins	Source species	Scores
Pheromone binding protein	*Helicoverpa armigera*	1 037
Pheromone binding protein-2	*Heliothis virescens*	500
Glyceraldehyde-3-phosphate dehydrogenase	*Plutella xylostella*	391
Ribosomal protein S20	*Spodoptera frugiperda*	373
Cytochrome oxidase subunit I	*H. armigera*	829
Allatotropin neuropeptide precursor (AT2)	*S. frugiperda*	109
Translationally controlled tumor protein (TCTP)	*Bombyx mori*	200
Humoral lectin prepropeptide	*B. mori*	369
Glycosylasparaginase alpha beta	*S. frugiperda*	71.9

4 DISCUSSION

4.1 Gqα may be involved in olfactory signal transduction

By two-hybrid technique, we determined more than ten positive proteins which may interact with Gqα. Among those proteins, pheromone binding proteins (PBPs) were closely related with the olfactory signal transduction. PBPs and their functions have been studied in many species, such as moths, bees, beetles and flies (Vogt *et al*., 1985; Danty *et al*., 1999; Jacquin-Joly *et al*., 2000, 2001; Kim and Smith, 2001; Nikonov *et al*., 2002; Wang *et al*., 2002, 2003, 2004). PBP was first identified in *Antheraea polyphemus*, which belongs to a multigene family – odor binding protein (OBP) family (Vogt and Riddiford, 1981; Blomquist and Vogt, 2003). OBP family consists of two subfamilies/classes: PBPs and general odorant binding proteins (GOBPs). PBPs were present at much higher level in males than in females, while GOBPs were present equally in both sexes (Vogt *et al*., 1991). The asymmetric distribution of PBPs between males and females was consistent with their roles in pheromone detection, and the equal distribution of GOBPs between males and females was helpful for the detection of general odorants (plant volatiles, non-pheromones) by both sexes (Blomquist and Vogt, 2003). Several studies indicated that odor-OBP complex stimulated olfactory receptors (ORs) (Prestwich *et al*., 1995; Ziegelberger, 1995). In this study, PBP was found to interact with Gqα, which would be a direct evidence to verify that Gqα plays roles in the olfactory signal transduction. But PBP was present in the extracellular fluid of the sensillum lumen and Gqα was the intracellular protein. The question is how the two proteins could interact with each other. One explanation may be that odor-OBP complex can bind with OR, which leads to the conformational change of OR, and thus OBP and Gqα are brought into proximity to interact with each other.

4.2 Gqα might play diverse roles in other signal transduction pathways

A Gα_{15} subunit was ever coexpressed with OR43a in frog oocytes which were then stimulated with applied odors. The oocytes responded to only four individual odors in the designed panel, cyclohexanol, cyclohexanone, benzaldehyde, and benzyl alcohol (Wetzel *et al*., 2001). In that experiment, no OBPs were applied to the system and no cell could express any OBP, indicating that the interaction between odor and OR did not need the involvement of OBP. From this point, we could presume that OBP-Gqα interaction may play some roles in other signal transduction pathways besides in the olfactory detection.

Moreover, G proteins not only are classically activated by 7TMRs (Cabrera-Vera *et al*., 2003), but also have emerged as non-canonical mediators of 7TMR-independent signaling pathways (Patel, 2004). It was found that Gqα was a ubiquitous protein, and its mRNA was expressed not only in antenna and other tissues of adult *H. armigera*, but also in its egg, larval and pupal stages (Su *et al.*, 2006). In our research, more than ten proteins such as PBP, GAPDH, TCTP, GA and ribosomal protein S20 were found to interact with Gqα, indicating that Gqα may play diverse roles in the signal transduction besides olfactory detection, which need further investigation.

4.3 Interactions between Gqα and the positive proteins need further study

Since interactions between Gqα and several proteins were only verified by the yeast two-hybrid system, we need to further test the interaction in yeast through cotransformation or yeast mating, or transferring the library insert from the AD to the DNA-BD vector. We can also test the interaction *in vitro* using co-immunoprecipitation (Co-IP).

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利用酵母双杂交技术筛选棉铃虫触角内与 G 蛋白 q 家族 α 亚基(Gqα)互作的蛋白

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摘要： G 蛋白在信号转导中起着重要的作用，许多研究表明 Gq 蛋白还具有其他功能。为了明确 Gqα 在信号转导中的具体作用，本研究以 Gqα 为诱饵，利用酵母双杂交技术对棉铃虫 *Helicoverpa armigera*（Hübner）触角 cDNA 文库进行筛选。结果表明：信息素结合蛋白(PBP)、3-磷酸甘油醛脱氢酶（GAPDH）、核糖体蛋白 S20、细胞色素氧化酶亚基Ⅰ、促咽侧体神经肽前体（AT2）、翻译控制的肿瘤蛋白（TCTP）、体液凝集素前多肽原和糖基化天冬酰胺酶等 10 多种蛋白与 Gqα 发生结合。据此推断：Gqα 可能在信号转导过程中发挥多种作用；在多种功能中，Gqα 最可能参与嗅觉信号传导中的信息素识别。这些研究结果可能为将来建立棉铃虫完整的信号网络提供基础。

关键词： 棉铃虫；Gqα；触角 cDNA 文库；酵母双杂交技术；信号转导；信息素结合蛋白

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